(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 March 2001 (22.03.2001)

PCT

(10) International Publication Number
WO 01/19851 A2

(51) International Patent Classification⁷: C07K 14/00

(21) International Application Number: PCT/CA00/01049

(22) International Filing Date: 18 September 2000 (18.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/154,668 16 September 1999 (16.09.1999) US

(71) Applicant (*for all designated States except US*): NEUROTROPHIC BIOSCIENCE INC. [CA/CA]; 96 Skyway Avenue, Etobicoke, Ontario M9W 4Y9 (CA).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): COMMISSIONG, John [CA/CA]; 3544 Redmond Road, Mississauga, Ontario L5B 3Y7 (CA). RAIBEKAS, Andrei, A. [CA/CA]; 255 Glenlake Avenue, Toronto, Ontario M6P 1G2 (CA).

(74) Agent: MBM & CO.; P.O. Box 809, Station B, Ottawa, Ontario K1P 5P9 (CA).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *Without international search report and to be republished upon receipt of that report.*

— *With (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: OPAMINERGIC NEURONAL SURVIVAL-PROMOTING FACTORS AND USES THEREOF

(57) Abstract: The invention features a pharmaceutical composition that includes arginine-rich protein and a pharmaceutically-acceptable excipient. The invention also features methods for treatment of a neurodegenerative disease, methods for improving neuronal survival during or following cell transplantation, methods for production of neurons for transplantation, and methods for identifying compounds that modulate or mimic arginine-rich protein's biological activity.

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DOPAMINERGIC NEURONAL SURVIVAL-PROMOTING FACTORS AND USES THEREOF

Background of the Invention

The invention relates to compositions and methods for increasing the survival of neurons.

The growth, survival, and differentiation of neurons in the peripheral and central nervous systems (PNS and CNS, respectively) are dependent, in part, on target-derived, paracrine, and autocrine neurotrophic factors. Conversely, the lack of neurotrophic factors is thought to play a role in the etiology of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease). In neuronal cultures, neurotrophic support is provided by co-culturing with astrocytes or by providing conditioned medium (CM) prepared from astrocytes. Astrocytes of ventral mesencephalic origin exert much greater efficacy in promoting the survival of ventral, mesencephalic dopaminergic neurons, compared with astrocytes from other regions of the CNS, such as the neostriatum and cerebral cortex. In chronic, mesencephalic cultures of 21 days *in vitro* (DIV) or longer, the percentage of dopaminergic neurons increases from 20% to 60%, coincident with proliferation of a monolayer of astrocytes. In contrast, in conditions in which the proliferation of astrocytes was inhibited, dopaminergic, but not GABAergic neurons, were almost eliminated from the cultures by 5 DIV. These results demonstrate the importance of homotypically-derived astrocytes for the survival and development of adjacent dopaminergic neurons, and suggest that mesencephalic astrocytes are a likely source of a physiological, paracrine neurotrophic factor for mesencephalic dopaminergic neurons.

The repeated demonstration that astrocytes secrete molecules that promote neuronal survival has made astrocytes a focus in the search for therapeutics to treat neurodegenerative diseases. Many laboratories have attempted to isolate astrocyte-derived neurotrophic factors, but have been hindered by a major technical problem: serum is an essential component of the medium for the optimal growth of primary astrocytes in culture, yet the presence of serum interferes with the subsequent purification of factors secreted into the conditioned medium.

Thus, there is a need to identify and purify new neurotrophic factors and to identify new methods to produce conditioned medium that are compatible with protein isolation techniques.

Summary of the Invention

We have isolated a spontaneously immortalized type-1 astrocyte-like cell line, referred to as ventral mesencephalic cell line-1 (VMCL-1). This cell line, deposited with the American Type Culture Collection (ATCC; Manassas, VA; ATCC Accession No:_____, deposit date, September 18, 2000), is derived from the ventral mesencephalon and retains the characteristics of primary, type-1 astrocytes, but grows robustly in a serum-free medium. The CM prepared from these cells contains one or more neuronal survival factors that increase the survival of mesencephalic dopaminergic neurons at least 3-fold, and promotes their development as well. The potency of this neurotrophic activity and its low degree of toxicity on dopaminergic neurons *in vitro* are distinguishing features of the activity of VMCL-1 CM. Moreover, using size fractionation techniques, we have identified activities that elute at about 14-16 kilodaltons, 18-21 kilodaltons, and 25-35 kilodaltons. The VMCL-1 immortalized cell line does not require serum for its growth and thus allows us to identify the VMCL-1 CM neuronal survival-promoting polypeptides.

Using a multi-step purification process, we identified arginine-rich protein (having a molecular weight of approximately 20 kilodaltons) as a protein that co-purified with the neuronal survival-promoting activity. As the protein and the activity co-purified through five purification steps, we conclude that this protein is one of the factors in the VMCL-1 CM having the desired neuronal survival-promoting activity.

Accordingly, in general, the invention features methods for increasing the survival of neurons (*e.g.*, dopaminergic neurons), as well as new polypeptides exhibiting such neuronal survival-promoting activity.

In a first aspect, the invention features a pharmaceutical composition that includes, as an active polypeptide, a substantially pure arginine-rich protein, and a pharmaceutically acceptable carrier. In one preferred embodiment, the arginine-rich protein is human arginine-rich protein (SEQ ID NO: 1).

In a second aspect, the invention features a substantially pure polypeptide having a molecular weight of about 14-16 kilodaltons that increases the survival of dopaminergic neurons.

In a third aspect, the invention features a substantially pure polypeptide having a molecular weight of about 18-21 kilodaltons that increases the survival of dopaminergic neurons.

In a fourth aspect, the invention features a substantially pure polypeptide having a molecular weight of about 25-35 kilodaltons that increases the survival of dopaminergic neurons.

The polypeptides of the present invention can be obtained from a glial cell line, such as VMCL-1 or another immortalized type-1 astrocyte cell line. In preferred embodiments of the first, second, third, or fourth aspect, the survival of dopaminergic neurons is increased at least three-fold. More preferably, survival is increased at least four-fold, while most preferably, survival is increased at least five-fold.

In another aspect, the invention features a method for increasing dopaminergic neuronal survival. The method includes contacting a dopaminergic neuron (either *in vitro* or *in vivo*) with a polypeptide of the first, second, third, or fourth aspect. A preferred polypeptide is human arginine-rich protein. Preferably, the survival of dopaminergic neurons is increased at least three-fold, more preferably at least four-fold, and most preferably at least five-fold.

In another aspect, the invention features a method for growing dopaminergic neurons for transplantation, including the step of culturing the neurons, or progenitor cells thereof, with an effective amount of a polypeptide of the first, second, third, or fourth aspect. As above, a preferred polypeptide is human arginine-rich protein. In preferred embodiments, the amount is sufficient to increase the survival of dopaminergic neurons by at least three-fold, by at least four-fold, or even by at least five-fold.

In still another aspect, the invention features a method of treating a patient having a disease or disorder of the nervous system, this method includes the step of administering to the patient a survival-promoting amount of a substantially purified arginine-rich protein.

In yet another aspect, the invention features another method for preventing dopaminergic neuronal cell death in a mammal. This method includes administering to the mammal a dopaminergic neuron survival-promoting amount of a substantially purified arginine-rich protein. A preferred mammal is a human.

The invention also features a method of transplanting cells into the nervous system of a mammal, including (i) transplanting cells into the nervous system of the mammal; and (ii) administering a dopaminergic neuronal survival-promoting amount of arginine-rich protein (e.g., human arginine-rich protein) to the mammal (e.g., a human) in a time window from four hours before transplanting of the cells to four hours after transplantation of the cells.

In preferred embodiments, the time window is from two hours before transplantation of the cells to two hours after transplantation of the cells.

The invention features another method of transplanting cells into the nervous system of a mammal. In this method, the cells are contacted with arginine-rich protein; and then transplanted into the nervous system of the mammal. Preferably, these two steps are performed within four hours of each other.

In yet another aspect, the invention features a method for the preparation of a dopaminergic neuronal survival-promoting polypeptide of the present invention, including culturing an immortalized type-1 astrocyte cell line under conditions permitting expression of the polypeptide.

In still another aspect, the invention features a substantially pure composition that includes a polypeptide that increases the survival of dopaminergic neurons, the polypeptide having a molecular weight of about 14-16 kilodaltons, about 18-21 kilodaltons, or about 25-35 kilodaltons.

Methods for treatment of diseases and disorders using the polypeptides or compositions of the invention are also features of the invention. For instance, a method of treatment of a disease or disorder of the nervous system (*e.g.*, Parkinson's disease) can be effected with the described polypeptides.

The invention also features a method for preventing dopaminergic neuronal cell death by administering an effective amount of a polypeptide of the invention. Such a medicament is made by administering the polypeptide with a pharmaceutically acceptable carrier.

The invention features the use of a polypeptide of the first, second, third, or fourth aspect in the manufacture of a medicament.

The invention further features the use of a polypeptide as defined herein: (1) to immunize a mammal for producing antibodies, which can optionally be used for therapeutic or diagnostic purposes; (2) in a competitive assay to identify or quantify molecules having receptor binding characteristics

corresponding to those of the polypeptide; (3) for contacting a sample with a polypeptide, as mentioned above, along with a receptor capable of binding specifically to the polypeptide for the purpose of detecting competitive inhibition of binding to the polypeptide; and (4) in an affinity isolation process, optionally affinity chromatography, for the separation of a corresponding receptor.

As mentioned above, the invention provides, from mammalian sources, new dopaminergic neuronal survival factors (*e.g.*, arginine-rich protein) that are distinguishable from known factors. These factors promote the survival of dopaminergic neurons. The invention also provides processes for the preparation of these factors, and a method for defining activity of these and other factors. Therapeutic application of the factors is a further significant aspect of the invention.

In other aspects, the invention features a polypeptide that increases the survival of dopaminergic neurons, the polypeptide having a molecular weight of about 14-16 kilodaltons or 25-35 kilodaltons (relative to proteins of known molecular weights, ranging from 15-102 kDa, run under the same conditions), as determined using a heparin sepharose CL-6B column (Sigma Chemicals, St. Louis, MO), and which has survival-promoting activity for dopaminergic neurons. It will be appreciated that the molecular weight range limits quoted are not exact, but are subject to slight variations depending upon the source of the particular polypeptide factor. A variation of about 10% would not, for example, be impossible for material from another source.

In another aspect, the invention features a pharmaceutical formulation that includes a polypeptide of the present invention formulated for pharmaceutical use, optionally together with an acceptable diluent, carrier or excipient and/or in unit dosage form. In using the factors of the invention, conventional pharmaceutical practice may be employed to provide suitable formulations or compositions. For example, it is preferred that any viral

pathogens that may be present with the substantially pure polypeptide be removed or inactivated, and that similar preventive measures are taken to remove any toxic compounds that are present with the substantially pure polypeptide. In one embodiment, the pharmaceutical formulation includes cells (*e.g.*, dopaminergic neurons or their progenitors) for transplantation.

In another aspect, the invention features a method of transplanting cells (*e.g.*, dopaminergic neurons or their progenitors) into the nervous system of a mammal. The method includes administering a polypeptide or composition of the present invention, in a pharmaceutically acceptable carrier to the mammal before, during, or after the cell transplantation.

It is preferred that the polypeptide or composition is administered to the mammal in a time window from four hours before transplantation to four hours after transplantation. More preferably, the time window is from two hours before transplantation to two hours after transplantation. It is understood that the polypeptide or composition does not have to be present during the entire time window for it to prevent or decrease cell death.

In a related aspect, the invention features another method of transplanting cells into the nervous system of a mammal. The method includes contacting the cells to be transplanted with a polypeptide or composition of the present invention, in a pharmaceutically acceptable carrier before cell transplantation.

It is preferred that the cells to be transplanted are contacted with the polypeptide or composition within four hours of transplantation, and, more preferably, within two hours of transplantation. It is understood that the polypeptide or composition does not have to be present for the entire time in order to prevent or decrease cell death following transplantation.

Parenteral formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets

or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are to be found in, for example, Remington: The Science and Practice of Pharmacy, (19th ed.) ed. A.R. Gennaro AR., 1995, Mack Publishing Company, Easton, PA. Formulations for parenteral administration may, for example, contain as excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes, biocompatible, biodegradable lactide polymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally.

The present factors can be used as the sole active agents, or can be used in combination with other active ingredients, *e.g.*, other growth factors which could facilitate neuronal survival in neurological diseases, or peptidase or protease inhibitors.

The concentration of the present factors in the formulations of the invention will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

In general terms, the factors of this invention may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v polypeptide for parenteral administration. General dose ranges are from about 1 mg/kg to about 1 g/kg of body weight per day; a preferred dose range is from about 0.01 mg/kg to 100 mg/kg of body weight per day. The preferred dosage

to be administered is likely to depend upon the type and extent of progression of the pathophysiological condition being addressed, the overall health of the patient, the make up of the formulation, and the route of administration.

As indicated above, dopaminergic neurons are, in large part, prevented from dying in the presence of the factors of the invention. Dopaminergic neurons of the mesencephalon die in patients having Parkinson's disease. The invention thus provides a treatment of Parkinson's disease. In addition, the use of the present factors in the treatment of disorders or diseases of the nervous system in which the loss of dopaminergic neurons is present or anticipated is included in the invention.

The invention also features screening methods for identifying factors that potentiate or mimic arginine-rich neuronal survival-promoting activity. In these screening methods for potentiators, the ability of candidate compounds to increase arginine-rich protein expression, stability, or biological activity is tested using standard techniques. A candidate compound that binds to arginine-rich protein may act as a potentiating agent. Alternatively, a mimetic (e.g., a compound that binds the arginine-rich protein receptor) is capable of acting in the absence of arginine-rich protein.

By "substantially pure" is meant that a polypeptide (*e.g.*, arginine-rich protein) has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is an arginine-rich protein that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure arginine-rich protein may be obtained, for example, by extraction from a natural source (*e.g.*, a neuronal cell), by expression of a recombinant nucleic acid encoding an arginine-rich protein, or by chemically synthesizing the protein. Purity can be measured by

any appropriate method, *e.g.*, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A polypeptide is substantially free of naturally associated components when it is separated from those contaminants that accompany it in its natural state. Thus, a polypeptide which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those which naturally occur in eukaryotic organisms but are synthesized in *E. coli* or other prokaryotes.

By "polypeptide" or "protein" is meant any chain of more than two amino acids, regardless of post-translational modification such as glycosylation or phosphorylation.

An arginine-rich protein that is a part of the invention includes a protein having dopaminergic neuronal survival-promoting activity and encoded by a nucleic acid that hybridizes at high stringency to the cDNA encoding human arginine-rich protein. A preferred arginine-rich protein is represented by the amino acid sequence of SEQ ID NO: 1.

Nucleic acids that are a part of the invention include those nucleic acids encoding proteins having dopaminergic neuronal survival-promoting activity and that hybridize at high stringency to the one of the strands of the cDNA encoding human arginine-rich protein (SEQ ID NO: 5). A preferred nucleic acid is represented by the nucleotide sequence of SEQ ID NO: 5.

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 50%, preferably 85%, more preferably 90%, and most preferably 95% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides,

preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "high stringency conditions" is meant hybridization in 2X SSC at 40°C with a DNA probe length of at least 40 nucleotides. For other definitions of high stringency conditions, see F. Ausubel et al., *Current Protocols in Molecular Biology*, pp. 6.3.1-6.3.6, John Wiley & Sons, New York, NY, 1994, hereby incorporated by reference.

By "polypeptide" or "factor" is meant a molecule having an activity that promotes the survival (or, conversely, prevents the death) of dopaminergic neurons in a standard cell survival assay. Compounds of the present invention have a molecular weight of about 14-16 kilodaltons, about 18-21 kilodaltons, or, alternatively, about 25-35 kilodaltons. Specifically excluded from the polypeptides of the invention are glial cell-derived neurotrophic factor (GDNF) (Lin et al., Science 260:1130-1132, 1993), neurturin (Kotzbauer et al., Nature 384:467-470, 1996), persephin (Millbrandt et al., Neuron 20:245-253, 1998), and artemin (Baloh et al., Neuron 21: 1291-1302, 1998).

By "composition" is meant a collection of polypeptides, including a polypeptide of the present invention.

By "pharmaceutically acceptable carrier" is meant a carrier that is physiologically acceptable to the treated mammal while retaining the therapeutic properties of the polypeptide with which it is administered. One exemplary pharmaceutically acceptable carrier is physiological saline solution. Other physiologically acceptable carriers and their formulations are known to one skilled in the art and described, for example, in Remington: The Science and Practice of Pharmacy, (19th ed.) ed. A.R. Gennaro AR., 1995, Mack Publishing Company, Easton, PA. It will be understood that viral pathogens and toxic compounds that may inadvertently be included with a polypeptide or composition if the present invention may be inactivated or removed using any suitable method known in the art.

By a compound having "dopaminergic neuronal survival-promoting activity" is the presence of the compound increases survival of dopaminergic neurons by at least two-fold in a neuronal survival assay (such as the one described herein) relative to survival of dopaminergic neurons in the absence of the compound. Preferably, the increase in the survival of dopaminergic neurons is by at least three-fold, more preferably by at least four-fold, and most preferably by at least five-fold. The assay can be an *in vitro* assay or an *in vivo* assay. Preferably, the assay is an in vitro assay (see the section entitled "cell viability assay," *infra*)

The present invention provides new methods and reagents for the prevention of neuronal cell death. The invention also provides pharmaceutical compositions for the treatment of neurological diseases or disorders of which aberrant neuronal cell death is one of the causes.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description of the Invention

We have discovered that a cell line of mesencephalic origin (termed "VMCL-1") secretes a factor that, in turn, promotes differentiation and survival of dopaminergic neurons. This cell line grows robustly in a serum-free medium. Moreover, the CM prepared from these cells contains one or more neuronal survival factors that increase the survival of mesencephalic dopaminergic neurons at least 3-fold, and promotes their development as well.

We purified, from the VMCL-1 cell line, a protein that we identified to be arginine-rich protein. We purified the this protein as follows. A 3 L volume of VMCL-1 conditioned medium was prepared, and subjected to five sequential steps of column chromatography. At each purification step, each column fraction was tested for biological activity in the bioassay referred to above. An estimate of the effect of each fraction on neuronal survival was done at 24 hour intervals, over a period of five days, and rated on a scale of 1-10. After the fifth purification step, the biologically active fraction and an adjacent inactive fraction were analyzed by SDS-PAGE. The results of the SDS-PAGE analysis revealed a distinctive protein band in the 20 kDa range in the lane from the active fraction. The "active" band was excised and subjected to tryptic digest, and the molecular mass and sequence of each peptide above background were determined by mass spectrometry analysis. The following two peptide sequences were identified: DVTFSPATIE (SEQ ID NO: 3) and QIDLSTVDL (SEQ ID NO: 4). A search of the database identified a match for human arginine-rich protein (SEQ ID NO: 1) and its mouse orthologue (SEQ ID NO: 2). The predicted protein encoded by the mouse EST sequence is about 95% identical to the predicted human protein. A search of the rat EST database revealed two sequences, one (dbEST Id: 4408547; EST name: EST348489) having significant homology at the amino acid level to the human and mouse proteins. The full-length rat sequence was not in the GenBank database.

Thus, arginine-rich protein is useful as a neurotrophic factor for the treatment of a neurodegenerative disease and for improving neuronal survival during or following transplantation into a human. Arginine-rich protein can also be used to improve the *in vitro* production of neurons for transplantation. In another use, arginine-rich protein allows for the identification of compounds that modulate or mimic its dopaminergic neuronal survival-promoting activity. The protein can also be used to identify its cognate receptor. Each of these uses is described in greater detail below.

Identification of molecules that modulate arginine-rich protein biological activity

The effect of candidate molecules on arginine-rich protein-mediated regulation of neuronal survival may be measured at the level of translation by using standard protein detection techniques, such as western blotting or immunoprecipitation with an arginine-rich protein-specific antibody.

Compounds that modulate the level of arginine-rich protein may be purified, or substantially purified, or may be one component of a mixture of compounds such as an extract or supernatant obtained from cells (Ausubel et al., *supra*). In an assay of a mixture of compounds, arginine-rich protein expression is measured in cells administered progressively smaller subsets of the compound pool (e.g., produced by standard purification techniques such as HPLC or FPLC) until a single compound or minimal number of effective compounds is demonstrated to arginine-rich protein expression.

Compounds may also be directly screened for their ability to modulate arginine-rich protein-mediated neuronal survival. In this approach, the amount of neuronal survival in the presence of a candidate compound is compared to the amount of neuronal survival in its absence, under equivalent conditions. Again, the screen may begin with a pool of candidate compounds, from which

one or more useful modulator compounds are isolated in a step-wise fashion. Survival-promoting activity may be measured by any standard assay.

Another method for detecting compounds that modulate the activity of arginine-rich protein is to screen for compounds that interact physically with arginine-rich protein. These compounds may be detected by adapting interaction trap expression systems known in the art. These systems detect protein interactions using a transcriptional activation assay and are generally described by Gyuris et al. (Cell 75:791-803, 1993) and Field et al., (Nature 340:245-246, 1989). Alternatively, arginine-rich protein or biologically active fragments thereof can be labeled with ^{125}I Bolton-Hunter reagent (Bolton et al. Biochem. J. 133: 529, 1973). Candidate molecules previously arrayed in the wells of a multi-well plate are incubated with the labeled arginine-rich protein, washed and any wells with labeled arginine-rich protein complex are assayed. Data obtained using different concentrations of arginine-rich protein are used to calculate values for the number, affinity, and association of arginine-rich protein with the candidate molecules.

Compounds or molecules that function as modulators of arginine-rich protein neuronal survival-promoting activity may include peptide and non-peptide molecules such as those present in cell extracts, mammalian serum, or growth medium in which mammalian cells have been cultured.

A molecule that modulates arginine-rich protein expression or arginine-rich protein-mediated biological activity such that there is an increase in neuronal cell survival is considered useful in the invention; such a molecule may be used, for example, as a therapeutic agent, as described below.

<u>Therapy</u>

The discovery of arginine-rich protein as a neurotrophic factor that promotes the survival of dopaminergic neurons allows for its use for the

therapeutic treatment of neurodegenerative diseases such as Parkinson's disease.

To add arginine-rich protein to cells in order to prevent neuronal death, it is preferable to obtain sufficient amounts of pure recombinant arginine-rich protein from cultured cell systems that can express the protein. Preferred arginine-rich protein is human arginine-rich protein, but arginine-rich protein derived from other animals (*e.g.*, pig, rat, mouse, dog, baboon, cow, and the like) can also be used. Delivery of the protein to the affected tissue can then be accomplished using appropriate packaging or administrating systems. Alternatively, small molecule analogs may be used and administered to act as arginine-rich protein agonists and in this manner produce a desired physiological effect.

Gene therapy is another potential therapeutic approach in which normal copies of the gene encoding arginine-rich protein (or nucleic acid encoding arginine-rich protein sense RNA) is introduced into cells to successfully produce arginine-rich protein. The gene must be delivered to those cells in a form in which it can be taken up and encode for sufficient protein to provide effective neuronal survival-promoting activity.

Retroviral vectors, adenoviral vectors, adenovirus-associated viral vectors, or other viral vectors with the appropriate tropism for neural cells may be used as a gene transfer delivery system for a therapeutic arginine-rich protein gene construct. Numerous vectors useful for this purpose are generally known (Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis and Anderson, BioTechniques 6:608-614, 1988; Tolstoshev and Anderson, Curr. Opin. Biotech. 1:55-61, 1990; Sharp, The Lancet 337: 1277-1278, 1991; Cornetta et al., Nucl. Acid Res. and Mol. Biol. 36: 311-322, 1987; Anderson, Science 226: 401-409, 1984; Moen, Blood Cells 17: 407-416, 1991; Miller et al., Biotech. 7: 980-990, 1989; Le Gal La Salle et al., Science 259: 988-990, 1993; and Johnson, Chest 107: 77S-83S, 1995).

Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med. 323: 370, 1990; Anderson et al., U.S. Patent No. 5,399,346). Non-viral approaches may also be employed for the introduction of therapeutic DNA into the desired cells. For example, arginine-rich protein may be introduced into a cell by lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84: 7413, 1987; Ono et al., Neurosci. Lett. 117: 259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Meth. Enzymol. 101:512, 1983), asialorosonucoid-polylysine conjugation (Wu et al., J. Biol. Chem. 263:14621, 1988; Wu et al., J. Biol. Chem. 264:16985, 1989); or, less preferably, micro-injection under surgical conditions (Wolff et al., Science 247:1465, 1990).

Gene transfer could also be achieved using non-viral means requiring infection *in vitro*. This would include calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes may also be potentially beneficial for delivery of DNA into a cell. Although these methods are available, many of these are of lower efficiency.

Many methods for introducing vectors into cells or tissues are available and equally suitable for use *in vivo*, *in vitro*, and *ex vivo*. For *ex vivo* therapy, vectors may be introduced into neural stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection and by liposome injections may be achieved using methods which are well known in the art. Transplantation of normal genes into the affected cells of a patient can also be useful therapy. In this procedure, a normal arginine-rich protein gene is transferred into neurons or glia, either exogenously or endogenously to the patient. These cells are then injected into the targeted tissue(s).

In the constructs described, arginine-rich protein cDNA expression can be directed from any suitable promoter (*e.g.*, the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated

by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in neural cells may be used to direct arginine-rich protein expression. The enhancers used could include, without limitation, those that are characterized as tissue- or cell-specific in their expression. Alternatively, if an arginine-rich protein genomic clone is used as a therapeutic construct (for example, following isolation by hybridization with the arginine-rich protein cDNA described herein), regulation may be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

RNA molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the backbone of the molecule. This concept can be extended in all of these molecules by the inclusion of nontraditional bases such as inosine, queosine, and wybutosine, as well as acetyl-, methyl-, thio-, and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases.

Another therapeutic approach within the invention involves administration of recombinant arginine-rich protein, either directly to the site of a potential or actual cell loss (for example, by injection) or systemically (for example, by any conventional recombinant protein administration technique).

An additional embodiment of the invention relates to the administration of a pharmaceutical composition, in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed above. Such pharmaceutical compositions may consist of arginine-rich protein, antibodies to arginine-rich protein, mimetics, or agonists of arginine-rich protein. The compositions may be administered alone or in combination with at least one

other agent, such as stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones.

In one example, arginine-rich protein is administered to a subject at the site that cells are transplanted. The administration of arginine-rich protein can be performed before or after the transplantation of the cells. Preferably, the two steps are within about four hours of each other. If desirable, arginine-rich protein can be repeatedly administered to the subject at various intervals before and/or after cell transplantation. This protective administration of arginine-rich protein may occur months or even years after the cell transplantation.

In addition to its administration to a human or other mammal, arginine-rich protein can also be used in culture to improve the survival of neurons during their production any time prior to transplantation. In one example, the cells to be transplanted are suspended in a pharmaceutical carrier that also includes a survival-promoting amount of arginine-rich protein. Arginine-rich protein can also be administered to the cultures earlier in the process (*e.g.*, as the neurons are first differentiating). It is understood that the neurons need not be primary dopaminergic neurons. Neurons (*e.g.*, dopaminergic neurons) that are differentiated, either *in vitro* or *in vivo*, from stem cells or any other cell capable of producing neurons can be cultured in the presence of arginine-rich protein during their production and maintenance.

While human arginine-rich protein is preferred for use in the methods described herein, arginine-rich protein has been identified in numerous species, including rat, mouse, and cow. One in the art will recognize that the identification of arginine-rich protein from other animals can be readily performed using standard methods. Any protein having dopaminergic neuronal survival-promoting activity and encoded by a nucleic acid that hybridizes to the

cDNA encoding human arginine-rich protein is considered part of the invention.

Diagnostics

Antibodies which specifically bind arginine-rich protein may be used for the diagnosis of conditions or diseases characterized by alterations in the levels of arginine-rich protein, or in assays to monitor patients being treated with arginine-rich protein. The antibodies useful for diagnostic purposes may be prepared in the same manner as those described above for therapeutics. Diagnostic assays for arginine-rich protein include methods which utilize the antibody and a label to detect arginine-rich protein in human body fluids or extracts of cells or tissues. The antibodies may be used with or without modification, and may be labeled by joining them, either covalently or non-covalently, with a reporter molecule. A wide variety of reporter molecules which are known in the art may be used, several of which are described herein.

A variety of protocols including ELISA, RIA, and FACS are known in the art for measuring arginine-rich protein art and provide a basis for diagnosing altered or abnormal levels of arginine-rich protein expression. Normal or standard values for arginine-rich protein expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to arginine-rich protein under conditions suitable for complex formation. The amount of standard complex formation may be quantified by various methods, but preferably by photometric, means. Quantities of arginine-rich protein expressed in subject, control and disease, samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.

The nucleic acid sequences encoding arginine-rich protein may also be used for diagnostic purposes. The nucleic acid sequences which may be used

include antisense RNA and DNA molecules, and oligonucleotide sequences. The nucleic acid sequences may be used to detect and quantitate gene expression in biopsied tissues in which expression of arginine-rich protein may be correlated with disease. The diagnostic assay may be used to distinguish between absence, presence, and excess expression of arginine-rich protein, and to monitor regulation of arginine-rich protein levels during therapeutic intervention.

Nucleic acid sequences encoding arginine-rich protein may be used for the diagnosis of conditions or diseases which are associated with altered expression of arginine-rich protein. The nucleic acid sequences encoding arginine-rich protein may be used in Southern or northern analysis, dot blot, or other membrane-based technologies; in PCR technologies; or in dip stick, pIN, ELISA or chip assays utilizing fluids or tissues from patient biopsies to detect altered arginine-rich protein expression. Such qualitative or quantitative methods are well known in the art.

The nucleotide sequences encoding arginine-rich protein may be labeled by standard methods, and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantitated and compared with a standard value. If the amount of signal in the biopsied or extracted sample is significantly altered from that of a comparable control sample, the nucleotide sequences have hybridized with nucleotide sequences in the sample, and the presence of altered levels of nucleotide sequences encoding arginine-rich protein in the sample indicates the presence of the associated disease. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or in monitoring the treatment of an individual patient.

In order to provide a basis for the diagnosis of disease associated with altered expression of arginine-rich protein, a normal or standard profile for

expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, which encodes arginine-rich protein, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with those from an experiment where a known amount of a substantially purified polynucleotide is used. Standard values obtained from normal samples may be compared with values obtained from samples from patients who are symptomatic for disease. Deviation between standard and subject values is used to establish the presence of disease.

Once disease is established and a treatment protocol is initiated, hybridization assays may be repeated on a regular basis to evaluate whether the level of expression in the patient begins to approximate that which is observed in the normal patient. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

The following examples are to illustrate the invention. They are not meant to limit the invention in any way.

<u>Example 1: Production and Analysis of VMCL-1 Cells</u>

The VMCL-1 cell line was made as follows. Rat E14 mesencephalic cells, approximately 2-3% of which are glioblasts, were incubated in medium containing 10% (v/v) fetal bovine serum for 12 hours and subsequently expanded in a serum-free medium, containing basic fibroblast growth factor (bFGF) as a mitogen. After more than 15 DIV, several islets of proliferating, glial-like cells were observed. Following isolation and passaging, the cells (referred to herein as VMCL-1 cells) proliferated rapidly in either a serum-free or serum-containing growth medium. Subsequent immunocytochemical analysis showed that they stained positive for two astrocytic markers, GFAP

and vimentin, and negative for markers of oligodendroglial or neuronal lineages, including A2B5, O4, GalC and MAP2. We have deposited the VMCL-1 cell line with the American Type Culture Collection (Manassas, VA; ATCC Accession No:_____, deposit date, September 18, 2000).

Serum-free CM, prepared from the VMCL-1 cells, caused increased survival and differentiation of E14 mesencephalic dopaminergic neurons in culture. These actions are similar to those exerted by CM derived from primary, mesencephalic type-1 astrocytes. The expression of mesencephalic region-specific genes (*e.g.*, wnt-1, en-1, en-2, pax-2, pax-5 and pax-8), was similar between VMCL-1 cells and primary, type-1 astrocytes of E14 ventral mesencephalic origin. In both, wnt-1 was expressed strongly, and en-1 less strongly, supporting an expression pattern expected of their mesencephalic origin. A chromosomal analysis showed that 70% of the cells were heteroploid, and of these, 50% were tetraploid. No apparent decline in proliferative capacity has been observed after more than twenty-five passages. The properties of this cell line are consistent with those of an immortalized, type-1 astrocyte.

The VMCL-1 cells have a distinctly non-neuronal, glial-like morphology, but lack the large, flattened shape that is typical of type-1 astrocytes in culture. Immunocytochemical analysis demonstrated that they stained positive for GFAP and vimentin, and negative for MAP2, A2B5 and O4. The cells were therefore not of the oligodendrocyte lineage. On the basis of a negative reaction to A2B5 and their morphological characteristics they were also not type-2 astrocytes. The classification that is supported by the immunocytochemical evidence is of type-1 astrocytes, although, as noted, these cells lack the classical morphological traits of primary type-1 astrocytes in culture.

Example 2: Action of VMCL-1 CM on E14 Dopaminergic Neurons in Culture

VMCL-1 CM was tested at 0, 5, 20 and 50% v/v, for its ability to influence survival and development of E14 mesencephalic dopaminergic neurons in culture. The cultures were primed with 10% fetal bovine serum (FBS) for 12 hours, then grown in a serum-free growth medium thereafter, until they were stained and analyzed after 7 DIV. There was a dose-dependent action of the CM on the increased survival of dopaminergic neurons. The CM increased survival by 5-fold. In contrast, there was no significant increase in non-dopaminergic neuronal survival. The profile of the biological action of this putative factor is quite different from that of CM derived from the B49 glioma cell line, the source of GDNF (Lin et al., Science 260: 1130-1132).

Example 3: Gene Expression Analysis

To further investigate the similarity between the VMCL-1 cell line and primary cultured astrocytes, we measured the expression of six marker genes characteristic of the mesencephalic region. Analysis of wnt-1, en-1, en-2, pax-2, pax-5, and pax-8 showed that all genes were expressed in both E13 and E14 ventral mesencephalon neural tissue, with the exception of pax-2, which was expressed at E13 but not E14 neural tissue. Both primary astrocytes and VMCL-1 cells expressed wnt-1 at levels comparable with those of E13 and E14 ventral mesencephalic neural tissue. The degree of expression of en-1 was similar in primary astrocytes and VMCL-1 cells, although at a lower level versus expression in E13 and E14 ventral mesencephalic tissue. In contrast, en-2, pax-5 and pax-8 were not expressed in either primary astrocytes or VMCL-1. Pax-2 was expressed in E13 but not E14 ventral mesencephalon, and in primary astrocytes, but not in VMCL-1.

Example 4: Chromosomal Analysis

Chromosomes were counted in 34 cells. Of these, 9 had a count of 42, the diploid number for rat. Of the 25 cells that were heteroploid, 12/25 or 48% were in the tetraploid range. Hyperdiploid (counts of 43-48) and hypodiploid (counts of 39-41) cells each accounted for 20% of the population, while 12% of the cells had structurally rearranged chromosomes.

The selective action of VMCL-1 CM in increasing the survival of dopaminergic neurons in culture provides a potential clinical use for the molecule(s) produced by this cell line. The lack of a toxic action of VMCL-1 CM at a concentration of 50% v/v indicates that the active, putative neurotrophic factor is not toxic. The action exerted by VMCL-1 CM mirrors almost exactly that of CM prepared from mesencephalic, primary type-1 astrocytes (Takeshima et al., J. Neurosci. 14: 4769-4779, 1994). A high degree of specificity of the putative factor from VMCL-1 for dopaminergic neurons is strongly indicated from the observation that general neuronal survival was not significantly increased, while the survival of dopaminergic neurons was increased 5-fold. We have demonstrated that primary type-1 astrocytes express GDNF mRNA, but have not detected GDNF protein by Western blot in the CM, at a sensitivity of 50 pg. Moreover, we have shown that under the present experimental conditions, the increased survival of dopaminergic neurons mediated by an optimal concentration of GDNF is never greater than 2-fold. These observations alone indicate that the factor responsible for the neurotrophic actions of VMCL-1 CM is not GDNF.

Example 5: Production of Type-1 Astrocyte-Conditioned Medium

E16 type-1 astrocyte CM (10 L) was filtered and applied to a heparin sepharose CL-6B column (bed volume 80 mL) which had previously been equilibrated with 20 mM Tris-HCl (Mallinckrodt Chemical Co. Paris, KY) pH 7.6 containing 0.2 M NaCl. After washing with equilibration buffer, bound

proteins were eluted from the column with a linear gradient of 0.2 M - 2 M NaCl in 20 mM Tris-HCl pH 7.6 (400 mL total volume, flow rate 100 mL/hr). Fractions were collected using a Pharmacia LKB fraction collector and absorbance was measured at 280 nm (Sargent-Welch PU 8600 UV/VIS Spectrophotometer). A 1 mL aliquot was taken from each fraction, pooled into groups of four (4 mL total volume) and desalted using Centricon-10® membrane concentrators (Millipore, Bedford, MA). Samples were diluted 1:4 in defined medium and bioassayed for dopaminergic activity. Active fractions were pooled (80 mL total volume) and then applied to a G-75 Sephadex® column (70 x 2.5 cm, Pharmacia Biotechnology Ltd., Cambridge, UK) which had been pre-equilibrated with 50 mM ammonium formate pH 7.4. Proteins were separated with the same buffer (flow rate, 75 mL/hr) and absorbance was measured at 280 nm. A 1 mL aliquot was taken from each fraction, pooled into groups of four (4 mL total volume), concentrated by lyopholyzation and reconstituted in 1 mL distilled water volume. Samples were then diluted 1:4 in defined medium for dopaminergic bioassay. Those with neurotrophic activity were further bioassayed as individual fractions.

An important distinguishing feature of VMCL-1 CM is that it promotes predominantly the survival of dopaminergic neurons, compared with the survival of GABAergic, serotonergic, and other neuronal phenotypes present in the culture. This claim of specificity is also made for GDNF. The results of extensive testing have demonstrated, however, that the VMCL-1-derived compound is not GDNF.

In order to express the protein, a pcDNA3-hARP expression construct, containing the human arginine-rich protein cDNA under the control of the CMV promoter, is transiently transfected into COS cells and the conditioned medium tested for dopaminergic neuronal survival-promoting activity. A myc tag can be inserted to facilitate purification and immunodetection of the recombinant protein.

Example 6: Isolation and Purification of a Protein having Dopaminergic Neuronal Survival-Promoting Activity

The purification protocol was performed as follows. All salts used were of the highest purity and obtained from Sigma Chemical Co. All buffers were freshly prepared and filtered via 0.2 μM filter (GP Express vacuum-driven system from Millipore)

Step 1: Heparin-Sepharose column chromatography (4°C)

Three liters of VMCL-1 conditioned medium was diluted with an equal volume of 20 mM sodium phosphate buffer, pH 7.2 at room temperature, filtered, and concentrated to 550 mL volume with 5K PREP/SCALE-TFF 2.5 ft^2 cartridge (Millipore). The concentrated material was loaded onto a 10 mL Heparin-Sepharose column assembled from 2 x 5 mL HiTrap Heparin columns (Pharmacia Biotech) and pre-equilibrated with at least 100 mL of 10 mM sodium phosphate buffer, pH 7.2 (buffer A). After the loading was complete, the column was washed with 100 mL of buffer A. A total of 10 fractions were eluted with buffer B (buffer A plus 1 M sodium chloride) in about 3 mL volumes each. A 300 μL sample was withdrawn for analysis.

Step 2: Superose 12 column chromatography (4°C)

All of the fractions from step 1 were pooled, then concentrated to 4.5 mL using Centricon Plus-20 concentrator (5,000 MWCO, Millipore), loaded onto 16 x 600 mm gel-filtration column packed with Superose 12 media (Prep Grade, Sigma Chemical Co.) and pre-equilibrated with at least 300 mL of 20 mM sodium phosphate buffer, pH 7.2 containing 0.6 M sodium chloride (GF buffer). The protein elution was conducted in GF buffer. Two milliliter fractions were collected and analyzed for activity. The active protein was eluted in a 15 mL volume after 84 ml of GF buffer was passed through the

column and corresponded to an approximately 20-30 kDa elution region based on the column calibration data obtained with protein standards (Bio-Rad).

Step 3: Ceramic Hydroxyapatite column chromatography (room temperature; FPLC system)

The active fractions from step 2 that corresponded to the 20-30 kDa elution region were pooled and concentrated to 7.5 mL, using a Centricon Plus-20 concentrator (5,000 MWCO), dialyzed overnight at 4°C against 2 L of 10 mM sodium phosphate buffer, pH 7.2 (buffer A) and loaded (via Superloop) onto a 1 mL pre-packed ceramic hydroxyapatite (Type I, Bio-Rad) column equilibrated with buffer A. After the excess of unbound protein (flow through) was washed off the column with buffer A, the linear gradient of buffer A containing 1.0 M NaCl was applied from 0 to 100%. One milliliter fractions were collected and analyzed for activity. The active protein was eluted as a broad peak within the region of gradient corresponding to 0.4-0.8 M NaCl concentration.

Step 4: Anion-exchange column chromatography (room temperature; FPLC system)

The fractions corresponding to the broad peak were pooled (total volume = 15 mL) and concentrated to 6 mL using Centricon Plus-20 (5,000 MWCO), dialyzed overnight at 4°C against 2 L of 20 mM Tris HCl buffer, pH 7.5 (buffer A), loaded (via Superloop) onto a 1 mL anion-exchange FPLC column (Uno, Bio-Rad), and equilibrated with buffer A. After the excess of unbound protein was washed off the column with buffer A, a linear gradient of 0-100% 1 M NaCl (in buffer A) was applied. One milliliter fractions were collected and analyzed for activity. The active protein was found in the flow-through (i.e., in the unbound protein fraction).

Step 5: BioSil 125 column chromatography (room temperature; HPLC system)

The active protein fraction from Step 4 (7 mL of total volume) was concentrated down to nearly zero volume (about 1 μL) using Centricon Plus-20 concentrator (5,000 MWCO) and reconstituted in 0.6 mL of 10 mM sodium phosphate buffer, pH 7.2. The reconstituted material (70 μL, analytical run) was loaded onto BioSil 125 HPLC gel-filtration column (Bio-Rad) equilibrated with 20 mM sodium phosphate buffer, pH 7.2 (GF buffer). The chromatography was conducted using HP 1100 Series HPLC system (Hewlett-Packard). The eluate was collected in 120 μL fractions and analyzed for activity and protein content (SDS-PAGE). The activity was found in fractions associated with the main 280-nm absorbance peak eluted from the column, which was represented by a 45-kDa protein according to SDS-PAGE analysis. Nevertheless, no activity was found in the side fractions of the 45-kDa protein peak, indicating that activity might be due to the presence of another protein that was co-eluted with 45 kDa protein, but at much lower concentration that could not be detected on the 12% SDS-PAGE silver-stained gel. Therefore, the remaining concentrated material from step 5 was further concentrated down to 80 μL volume using a Centricon-3 concentrator (Millipore), and 60 μL was loaded and separated on the column at the same conditions as for the above-described analytical run. Aliquots of 8 μL were taken from each 120 μL fraction of the eluate and analyzed by SDS-PAGE (12% gel) combined with silver staining. This analysis indicated that another two additional proteins (having molecular weights of about 18 and 20 kDa) were associated with the active fractions and co-eluted with the major 45-kDa protein. The active fractions were dialyzed against 1 L of ammonium acetate buffer, pH 8.0 (4°C) and combined to create two active pools, P-1 and P-2, such that P-1 contained the 20 kDa protein and the 45 kDa protein, and P-2 contained the 18 kDa protein and the 45 kDa protein. Each pool was dried down on SpeedVac vacuum concentrator (Savant) and separately reconstituted

in 15 μL 0.1 M ammonium acetate buffer, pH 6.9. Aliquots were withdrawn from each sample and assayed for activity. Additionally, 1 μL aliquots were subjected to 12% SDS-PAGE analysis followed by silver staining.

The results of the foregoing analysis clearly indicated that P-1, but not P-2, contained the desired survival-promoting activity. In the next step, both P-1 and P-2 were dried on SpeedVac, reconstituted (each) in 10 μL of freshly prepared SDS-PAGE reducing sample buffer (Bio-Rad), incubated for one minute in a boiling water bath and loaded onto a 12% SDS-PAGE gel. After electrophoresis was complete, the gel was fixed in methanol/acetic acid/water solution (50:10:40) for 40 minutes at room temperature, washed three times with nanopure water, and stained overnight with GelCode Blue Stain Reagent (Pierce) at room temperature. After staining was completed, and the GelCode solution was washed off the gel with nanopure water, the visible protein bands corresponding to the 45 kDa protein (both P-1 and P-2) and the 20 kDa protein (P-1 only) were excised from the gel with a razor blade. Each gel slice containing a corresponding band was placed in a 1.5 mL microcentrifuge tube until the time of in-gel digestion.

Example 7: Analysis of In-gel Digested Fragments by nESI-MS/MS

The protein gel bands were incubated with 100 mM ammonium bicarbonate in 30% acetonitrile (aq.) at room temperature for 1 hour in order to remove the colloidal Coomassie blue stain. The destaining solution was replaced a number of times until the dye was completely removed. The gel pieces were then covered with deionized water (~ 200 μL) and shaken for 10 minutes. The gel pieces were dehydrated in acetonitrile and, after removing the excess liquid, were dried completely on a centrifugal evaporator. The gel bands were rehydrated with 20 μL of 50 mM ammonium bicarbonate, pH 8.3, containing 200 ng of modified trypsin (Promega, Madison, WI). The gel pieces were covered with 50 mM ammonium bicarbonate, pH 8.3

(approximately 50 µL), and were incubated overnight at 37°C. The digest solutions were then transferred to clean eppendorf tubes and the gel pieces were sonicated for 30 minutes in 50-100 µL of 5% acetic acid (aq). The extract solutions were combined with the digest solutions and evaporated to dryness on a centrifugal evaporator.

The in-gel digest extracts were first analyzed by matrix-assisted laser desorption ionization - time of flight mass spectrometry (MALDI-TOFMS) using a Voyager Elite STR MALDI-TOFMS instrument (Applied Biosystems Inc., Framingham, MA). The extracts were dissolved in 5 µL of 50% acetonitrile, 1% acetic acid. Dihydroxybenzoic acid was used as the matrix and spectra were acquired in positive ion, reflectron mode. Approximately one fifth of each sample was used for this analysis. These spectra provided the masses of the peptides in the digest extracts which were then used to search an in-house, non-redundant protein sequence database, a process called peptide mass fingerprinting. The remainder of the samples were used for peptide sequencing analysis by nanoelectrospray ionization - tandem mass spectrometry (nESI-MS/MS). The extracts were first desalted using C18 ZipTips (Millipore) and redissolved in 75% methanol (aq.), 0.1% acetic acid (5 µL). Approximately one half of the samples were loaded into nanoelectrospray glass capillaries (Micromass). nESI-MS/MS analyses were carried out using a Q-Star quadrupole time-of-flight hybrid mass spectrometer (PE SCIEX, Concord, ON). All MS/MS analyses were carried out in positive ion mode. The collision gas was nitrogen and the collision energy was 40-60 eV. MS/MS spectra were typically acquired every second over a period of two minutes. The MS/MS spectra were used to search an in-house non-redundant protein sequence database using partial sequence tags (i.e., only the peptide mass and a few fragment ions are used to search the database). If the protein was not identified by this procedure then the amino acid sequences of two or more

peptides were determined as fully as possible from the MS/MS spectra. These sequences were used to carry out BLAST searches on NCBI's protein, nucleotide and EST sequence databases.

The results of the analysis identified the 45 kDa protein in both P-1 (active) and P-2 (inactive) as glia-derived nexin, and the 20 kDa protein in P-1 as arginine-rich protein. Therefore, arginine-rich protein is likely to be a main protein that is responsible for activity observed in P-1, while the necessity of the presence of nexin for activity cannot be excluded.

Example 8: Generation of Immortalized Cell Line from Human Mesencephalic Tissue

Using the methods described herein, one may produce a type-1 astrocyte-derived cell line, having the same or similar neuronal survival-promoting activity, from aborted human tissue. In humans, the corresponding gestational age of E14 is approximately 9-10 weeks, although other ages are also likely to be successful. The human compound is identified using standard protein purification techniques, as described herein.

To induce a spontaneous immortalization of human fetal astrocytes, ventral mesencephalic tissue is dissected from human fetal brain. The dissection is preferably performed under sterile conditions in salt solution (*e.g.*, Hank's balanced salt solution (HBSS)), at pH 7.4. The ventral mesencephalon (VM) with the floor plate intact, is localized, micro-dissected in a culture dish in fresh, salt solution, thoroughly cleared of non-neural tissue, and stored in salt solution. After tissue collection, the salt solution is removed, and the tissue rinsed with two changes of growth medium (*e.g.*, N2), then dispersed in 2.0 mL of growth medium, which is used in all subsequent procedures. The tissue is then triturated. About 10-15 strokes are needed to disperse the cells completely. The cells are centrifuged (1,000 rpm, 2 minutes), the medium aspirated, and the pellet dispersed in growth medium. The cells are counted

using a hemocytometer, and the density adjusted approximately 2.5 x 10^5 cells/mL. The cells are then dispersed in cultures dishes previously coated polyornithine (15 mg/mL) and fibronectin (1.0 mg/mL), at a density of 5.0 x 10^4 cells/cm^2. The dishes are transferred to the incubator (37°C, 5% CO_2, 100% humidity). bFGF (10 ng/mL) is added daily, and the medium changed every second day. At 8-12 DIV, when the cultures are approximately 50% confluence, the cells are disturbed for 5 days. These conditions have been shown previously to cause a small percentage of the expanding astrocytes to become spontaneously immortalized.

Alternatively, a human mesencephalic type-1 astrocyte cell line may be established from primary cultures by transforming the cells with a DNA construct containing the oncogenic early region of SV40, under the transcriptional control of a human GFAP promoter, and a selectable marker (*e.g.*, pPGK-neo, which contains the murine phosphoglycerate kinase gene promoter). The transformants are selected with G418 and cloned. It has been previously demonstrated that other transformed astrocytes retained characteristics consistent with the phenotype of type 1 astrocytes, including GFAP immunoreactivity and a high affinity uptake mechanism for GABA that is inhibitable by beta alanine (Radany et al. Proc. Natl. Acad. Sci. U S A 89:6467-6471, 1992).

The foregoing results were obtained with the following methods.

Mesencephalic Cultures

Primary mesencephalic cell culture was prepared from timed-pregnant Sprague-Dawley rats (Taconic Farms; Germantown, NY). as described previously (Shimoda et al., Brain Res. 586:319-323, 1992 ; Takeshima et al., J. Neurosci. 14:4769-4779, 1994; Takeshima et al., Neuroscience. 60:809-823, 1994; Takeshima et al., J. Neurosci. Meth. 67:27-41, 1996). The dissected tissue was collected and pooled in oxygenated, cold (4°C), HBSS or medium

containing 10% fetal bovine serum (Biofluids Laboratories, Rockville, MD), depending on the purpose of the experiment. Pregnant rats were killed by exposure to CO_2 on the fourteenth gestational day (i.e., E14), the abdominal region was cleaned with 70% EtOH, a laparotomy was performed, and the fetuses collected and pooled in cold Dulbecco's phosphate-buffered saline (DPBS), pH 7.4, without Ca^{2+} or Mg^{2+}. The intact brain was then removed, a cut was made between the diencephalon and mesencephalon, and the tectum slit medially and spread out laterally. The ventral, medial 1.0 mm^3 block of tissue comprising the mesencephalic dopaminergic region was isolated. Dissected tissue blocks were pooled in cold (4°C), oxygenated medium. The tissue was triturated without prior digestion. Alternatively, the cells were incubated in L-15 growth medium containing papain (Sigma Chemical Co.), 10 U/mL, at 37°C, for 15 minutes, washed (3 x 2 mL) with medium, and triturated using only the needle and syringe. The dispersed cells were transferred to 1.5 mL Eppendorf tubes (1.0 mL / tube), and centrifuged at ~600 g for 2 minutes. The use of higher centrifugation speeds for longer periods results in contamination of the cultures with debris and, as a result, suboptimal growth of the cells. The medium was carefully aspirated, and the cells resuspended in fresh medium and counted using a hemocytometer. All procedures, from laparotomy to plating were completed within 2 hours. In a typical experiment, one litter of 10-15 fetuses yielded 1.0×10^5 cells/fetus, or 1.0×10^6 - 1.5×10^6 cells/litter.

Mesencephalic Microisland Cultures

To make mesencephalic microisland cultures, cells were prepared as described above, and resuspended at a final density of 5.0×10^5/mL. A 25 μL droplet of the suspension (1.25×10^4 cells) was plated using a 100μL pipette onto 8-well chamber slides coated with poly-D-lysine (50 μg/mL). The area of the droplet was ~12.5 mm^2, for a final mean cell density of $1.0 \times 10^5/cm^2$. The

droplet was dispensed uniformly, and the pipette tip withdrawn vertically, to avoid smearing. The area occupied by the microisland culture remained uniform for the duration of the culture. The cultures were incubated for 30 minutes at 37°C, in 5% CO_2 at 100% humidity, to allow the cells to attach, and 375 µL of growth medium was then added to each well. The medium was changed after the first 12 hours, and approximately half of the medium was changed every second day thereafter.

Cell Viability Assay

A two-color fluorescence cell viability assay kit (Live/Dead Viability/Cytotoxicity Assay Kits, #L-3224, Molecular Probes, Inc., Eugene, OR) was used to identify live and dead cells prior to plating and in cultures. Live and dead cells fluoresced green and red, respectively, giving two positive indicators of viability. Ethidium homodimer and calcein-AM were diluted with DPBS to give final concentrations of 3.8 µM and 2.0 µM, respectively. Evaluation of cell viability was done before plating. A cell suspension was incubated for 15 minutes with an equal volume of dye (typically 20 µL) on glass slides at room temperature in a dark, humid chamber, coverslipped, and then examined with a fluorescent microscope using FITC optics. Cell viability just before plating was about 95%.

Culture Medium

The serum-free medium used consisted of equal volumes of Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 (Gibco, Grand Island, N.Y.; 320-1320AJ), 1.0 mg/mL bovine albumin fraction V (Sigma Chemical Co.; A-4161), 0.1 µg /mL apo-transferrin (Sigma; T-7786), 5 µg/mL insulin (Sigma; I-1882), 30 nM L-thyroxine (Sigma; T-0397) , 20 nM progesterone (Sigma; P-6149), 30 nM sodium selenite (Sigma; S-5261), 4.5 mM glutamine (Gibco,

320-5039AF), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco; P-100-1-91).

Preparation of Conditioned Medium from VMCL-1 Cell Line

In preparing conditioned medium from the VMCL-1 cell line, 2.0 x 10^6 cells were plated in a 15 cm uncoated culture dish, in 20 mL of growth medium containing 1.0% of FBS. At 80% confluence, the medium was aspirated and the cells washed once with serum-free medium. 20 mL of serum-free N2 medium without albumin was added, and conditioning allowed to continue for 48 hours. During this time, the cells usually expanded to 100% confluence. The medium was aspirated, pooled in 50 mL tubes, centrifuged (15,000 rpm for 20 minutes) and subsequently pooled in a 1.0 L plastic bottle. Usually 5 mL of each batch of CM was filter-sterilized using a 0.22 μm filter, stored at aliquots of 5 mL, at -70°C, and used to determine neurotrophic potency, before being pooled with the larger store of CM. If desired, VMCL-1 CM can be made in large quantities using standard industrial cell culture techniques known to those in the art.

Production of Conditioned Medium for Type-1 Astrocytes

Type-1 astrocytes were prepared as follows. E16 rat fetal brain stem was dissected in cold DPBS, and the mesencephalic region transferred to astrocyte culture medium (DMEM/Ham's F-12, 1:1, 15% FBS, 4.0 mM glutamine, 30 nM sodium selenite, penicillin, and streptomycin). Cells were dispersed by trituration in 2 mL of fresh medium using an 18-gauge needle fitted to a syringe. Cells were centrifuged for 5 minutes at 2,000 rpm in a centrifuge, re-suspended in medium, and triturated again. The final cell pellet was dispersed and plated at a density of 1 x 10^6 cells / 75 cm^2 flask in 15 mL of medium. Cells were incubated at 37°C in an atmosphere of 5% carbon dioxide and 95% air for 24 hours, and unattached cells were removed by aspiration.

Cells were cultured for an additional nine days, and flasks were then shaken vigorously for 16 hours to remove any contaminating cell types. Astrocyte monolayers were washed three times with DPBS, trypsinized and replated (density of 1 x 10^6 cells/flask). At this time, a small proportion of the cells were plated onto eight-well chamber slides (Nunc Inc., Naperville, IL); these sister cultures were treated as described for the flask cultures. At confluence, the medium was replaced with medium containing 7.5% FBS and cells were incubated for 48 hours. At the next exchange, defined serum-free medium (DMEM/Ham's F-12, 1:1, 4.0 mM glutamine, 30 nM sodium selenite, penicillin 100 U/mL and streptomycin 100 mg/mL) was added and cells were incubated for a further 48 hours. Medium was replaced and, after five days, conditioned medium was harvested and mixed with leupeptin (10 mM: Bachem, Torrance, CA) and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (1.0 mM: ICN Biochemicals, Aurora, OH) to inhibit proteolysis. At the time of harvesting, astrocyte monolayers cultured on chamber slides were immunostained in order to assess the culture phenotype.

Culturing of VMCL-1 Cells

In culturing VMCL-1 and preparing VMCL-1 CM, 2.0 x 10^6 cells were plated in a 15-cm uncoated culture dish, in 20 mL growth medium initially containing 10% FBS. At 80% confluence, the medium was aspirated and the cells washed once with serum-free medium. Usually 20 mL of serum-free medium without albumin was added, and conditioning allowed to continue for 48 hours. N2 medium proved to be particularly suitable for use to collect conditioned medium. During these 48 hours, the cells usually expanded to 100% confluence. The medium was aspirated, pooled in 50 mL tubes, centrifuged (15,000 rpm, 20 min) and pooled in a 1.0 L plastic bottle. Approximately 5 mL of each batch of CM was sterilized using a 0.22 mm filter, stored at aliquots of 0.5 mL, at -70°C, and used to determine neurotrophic

potency, before being pooled with the larger store of CM. The VMCL-1 cell line has now been passaged greater than 50 times.

Immunocytochemistry

For MAP2 and TH immunocytochemistry, the cultures were washed (2 x 250 μL) with cold DPBS, fixed with 4% formaldehyde in PBS for 10 minutes, permeabilized using 1% CH_3COOH/95% EtOH at -20°C, for 5 minutes, and then washed (3 x 250 μL) with PBS. Non-specific binding was blocked with 1% bovine serum albumin in PBS (BSA-PBS) for 15 minutes. Anti-TH antibody (50 μL) (Boehringer-Mannheim, Indianapolis, IN), or anti-MAP2 antibody (Boehringer-Mannheim) was applied to each well, and the slides incubated in a dark humid box at room temperature for 2 hours. Control staining was done using mouse serum at the same dilution as the anti-TH antibody. After washing (2 x 250 μL) with PBS, anti-mouse IgG-FITC (50 μL) was applied, and the slides incubated for an additional 1 hour. After washing with PBS (2 x 250 μL), excess fluid was aspirated, the chamber walls removed, and a single drop of VectaShield mounting medium (Vector Laboratories, Burlingame, CA) applied, followed by a cover glass, which was sealed with nail polish. In some experiments, TH was identified using biotinylated, secondary antibodies, and the nickel-enhanced, diaminobenzidine (DAB) reaction product was developed using the biotinylated peroxidase-avidin complex (ABC kit; Vector Laboratories).

For glial fibrillary acidic protein (GFAP, Boehringer-Mannheim, #814369), fixation and permeabilization were done in one step using 5% CH_3COOH/95% C_2H_5OH at -20°C. The subsequent procedures were the same as those used to visualize TH. For A2B5 and O4, the cultures were washed with cold DPBS (2 x 250 μL) and blocked with 1% BSA-PBS for 10 minutes. The A2B5 antibody (50 μL) was applied to each well, and incubated for 1 hour. After washing with DPBS (2 x 250 μL), the secondary antibody, anti-IgM-FITC, was applied for 30 minutes. The cells were then washed with

DPBS (2 x 250μL). To counter-stain cell nuclei, cells were incubated with 0.5 μg/mL of nucleic acid dye H33258 (Hoechst, Kansas City, MO) in 10 mM sodium bicarbonate for 15 minutes at room temperature, then rinsed in PBS for 2 x 10 minutes. After a final washing with cold DPBS (2 x 250 μL), they were mounted as described above.

RT-PCR Analysis

Total RNA was extracted from rat E13 or E14 ventral mesencephalic tissue or from 1 x 10^9 astrocytes or from 1 x 10^9 VMCL-1 cells using RNA-STAT reagent (TelTest, University of Maryland, Baltimore, MD). First strand cDNA was generated from RNA and amplified by polymerase chain reaction using the manufacturer's procedures.

Reaction products were resolved by 2% agarose gel electrophoresis to determine size and relative abundance of fragments. PCR results for β-actin and GAPDH were included as controls to confirm equal loading of cDNA.

Chromosomal Analysis

The cells were grown in DMEM/F-12 1:1 medium supplemented with 2.5% FBS, D-glucose (2.5 g/L) and ITS supplement, diluted 1:100. Twenty-four hours later, subcultures at metaphase stage were arrested with colchicine (10 μg/mL). The cells were trypsinized and subjected to hypotonic shock (75 mM KCl). The cells were then fixed in three changes of MeOH/CH_3COOH, 3:1, and air-dried. The cells were then stained using 4% Geisma, and microscopically examined.

<u>Deposit</u>

Applicant has made a deposit of at least 25 vials containing cell line VMCL-1 with the American Type Culture Collection, Manassas VA, 20110 U.S.A., ATCC Deposit No. _______. The cells were deposited with the ATCC on September 18, 2000. This deposit of VMCL-1 will be maintained in the

ATCC depository, which is a public depository, for a period of 30 years, or 5 years after the most recent request, or for the effective life of the patent, whichever is longer, and will be replaced if it becomes nonviable during that period. Additionally, Applicant has satisfied all the requirements of 37 C.F.R. §§1.801-1.809, including providing an indication of the viability of the sample. Applicant imposes no restrictions on the availability of the deposited material from the ATCC. Applicant has no authority, however, to waive any restrictions imposed by law on the transfer of biological material or its transportation in commerce. Applicant does not waive any infringement of its rights granted under this patent.

Other Embodiments

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the invention.

What is claimed is:

1. A pharmaceutical composition comprising: (i) a substantially pure arginine-rich protein; and (ii) a carrier that is pharmaceutically acceptable for administration to the central nervous system.

2. The pharmaceutical composition of claim 1, wherein said arginine-rich protein is human arginine-rich protein.

3. The pharmaceutical composition of claim 1, wherein said pharmaceutically acceptable carrier is a liposome.

4. A pharmaceutical composition comprising: (i) a substantially pure arginine-rich protein;(ii) a pharmaceutically acceptable carrier; and (iii) a neural cell.

5. The pharmaceutical composition of claim 4, wherein said neural cell is a neuron, a neural stem cell, or a neuronal precursor cell.

6. A method for increasing survival of dopaminergic neurons, said method comprising the step of contacting said dopaminergic neurons with a survival-promoting amount of a substantially purified arginine-rich protein.

7. The method of claim 6, wherein said arginine-rich protein is human arginine-rich protein.

8. A method for growing dopaminergic neurons for transplantation, said method comprising the step of culturing said neurons, or progenitor cells thereof, with a survival-promoting amount of a substantially purified arginine-rich protein.

9. The method of claim 8, wherein said arginine-rich protein is human arginine-rich protein.

10. The method of claim 8, wherein said arginine-rich protein is administered with a pharmaceutically acceptable excipient.

11. A method of treating a patient having a disease or disorder of the nervous system, said method comprising the step of administering to said patient a survival-promoting amount of a substantially purified arginine-rich protein.

12. A method for preventing dopaminergic neuronal cell death in a mammal, said method comprising administering to said mammal a survival-promoting amount of a substantially purified arginine-rich protein.

13. A method of transplanting cells into the nervous system of a mammal, comprising (i) transplanting cells into the nervous system of said mammal; and (ii) administering a survival-promoting amount of arginine-rich protein to said mammal in a time window from four hours before said transplantation of said cells to four hours after said transplantation of said cells.

14. The method of claim 13, wherein said mammal is a human.

15. The method of claim 13, wherein said arginine-rich protein is human arginine-rich protein.

16. The method of claim 13, wherein said time window is from two hours before said transplantation of said cells to two hours after said transplantation of said cells.

17. A method of transplanting cells into the nervous system of a mammal, said method comprising the steps of:

(a) contacting said cells with arginine-rich protein; and

(b) transplanting said cells into the nervous system of said mammal.

18. The method of claim 17, wherein step (a) and step (b) are performed within four hours of each other.

19. The method of claim 17, wherein said mammal is a human.

20. The method of claim 11, wherein said arginine-rich protein is human arginine-rich protein.

21. A substantially pure compound having a molecular weight of about 14-16 kilodaltons, wherein said compound increases the survival of dopaminergic neurons.

22. A substantially pure compound having a molecular weight of about 25-35 kilodaltons, wherein said compound increases the survival of dopaminergic neurons.

23. The compound of claim 21 or 22, wherein said compound is obtained from a glial cell line.

24. The compound of claim 23, wherein said glial cell line is VMCL-1.

25. A method for increasing dopaminergic neuronal survival, said method comprising contacting a dopaminergic neuron with a compound of claim 21 or 22.

26. A method for growing dopaminergic neurons for transplantation, said method comprising culturing said neurons, or progenitor cells thereof, with a compound of claim 21 or 22.

27. A method for the preparation of a compound of claim 21 or 22, said method comprising culturing an immortalized type-1 astrocyte cell line under conditions permitting expression of said compound.

28. A substantially pure composition comprising a compound that increases the survival of dopaminergic neurons, said compound having a molecular weight of about 14-16 kilodaltons or a molecular weight of about 25-35 kilodaltons.

29. A method for preventing dopaminergic neuronal cell death in a human, said method comprising administering to said human an effective amount of a compound of claim 21 or 22.

30. Use of a compound of claim 21 or 22 for the manufacture of a medicament.

31. A pharmaceutical formulation comprising a compound of claim 21 or 22 formulated for pharmaceutical use, optionally together with an acceptable diluent, carrier or excipient and/or in unit dosage form.

32. A method of transplanting cells into the nervous system of a mammal, said method comprising administering a compound of claim 21 or 22 in a pharmaceutically acceptable carrier to said mammal before, during, or after said cell transplantation, wherein said compound is administered to said

mammal in a time window from four hours before transplantation to four hours after transplantation.

33. A method of transplanting cells into the nervous system of a mammal, said method comprising contacting said cells with a compound of claim 21 or 22 within four hours of transplantation.

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